

3-20-2003

## Inhibition of heparanase activity and heparanase-induced angiogenesis by suramin analogues

Dario Marchetti  
*School of Veterinary Medicine*

Jane Reiland  
*School of Veterinary Medicine*

Brad Erwin  
*School of Veterinary Medicine*

Madhuchhanda Roy  
*School of Veterinary Medicine*

Follow this and additional works at: [https://digitalcommons.lsu.edu/biosci\\_pubs](https://digitalcommons.lsu.edu/biosci_pubs)

---

### Recommended Citation

Marchetti, D., Reiland, J., Erwin, B., & Roy, M. (2003). Inhibition of heparanase activity and heparanase-induced angiogenesis by suramin analogues. *International Journal of Cancer*, 104 (2), 167-174.  
<https://doi.org/10.1002/ijc.10930>

This Article is brought to you for free and open access by the Department of Biological Sciences at LSU Digital Commons. It has been accepted for inclusion in Faculty Publications by an authorized administrator of LSU Digital Commons. For more information, please contact [ir@lsu.edu](mailto:ir@lsu.edu).

## INHIBITION OF HEPARANASE ACTIVITY AND HEPARANASE-INDUCED ANGIOGENESIS BY SURAMIN ANALOGUES

Dario MARCHETTI\*, Jane REILAND, Brad ERWIN and Madhuchanda ROY

Department of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA, USA

**Heparanase, a heparan sulfate-specific endo- $\beta$ -D-glucuronidase, plays an important role in tumor cell metastasis through the degradation of extracellular matrix heparan sulfate proteoglycans (ECM HSPG). Heparanase activity correlates with the metastatic propensity of tumor cells. Suramin, a polysulfonated naphthylurea, is an inhibitor of heparanase with suramin analogues shown to possess antiangiogenic and antiproliferative properties. We investigated the effects of selected suramin analogues (NF 127, NF 145 and NF 171) on heparanase activity and heparanase-driven angiogenesis. Studies of the ability of cellular extracts and purified heparanase from human, highly invasive and brain-metastatic melanoma (70W) cells revealed that heparanase expressed by these cells was effectively inhibited by suramin analogues in a dose-dependent manner. These analogues possessed more potent heparanase inhibitory activities than suramin: The concentrations required for 50% heparanase inhibition ( $IC_{50}$ ) were 20–30  $\mu$ M, or at least 2 times lower than that for suramin. One hundred percent inhibition was observed at concentrations of 100  $\mu$ M and higher. Of relevance, these compounds significantly decreased (i) the invasive capacity of human 70W cells by chemoinvasion assays performed with filters coated with purified HSPG or Matrigel<sup>TM</sup>, and (ii) blood vessel formation by *in vivo* angiogenic assays, thus linking their antiangiogenic properties with impedance of heparanase-induced angiogenesis. Specifically, inhibition of invasion by NF 127, NF 145 and NF 171 was found at 10  $\mu$ M concentrations of compounds with a significant decrease of invasive values at concentrations as low as 1.5  $\mu$ M. In addition, NF 127, NF 145 and NF 171 promoted nearly complete inhibition of heparanase-induced angiogenesis at values ranging from 236  $\mu$ M (for NF 145) to 362  $\mu$ M (for NF 127). These results further emphasize the importance of heparanase in invasive and angiogenic mechanisms and the potential clinical application of heparanase inhibitors such as suramin analogues in cancers and angiogenesis-dependent diseases.**

© 2003 Wiley-Liss, Inc.

**Key words:** heparanase; brain-metastatic melanoma; suramin analogues invasion; angiogenesis; suramin

Tumor cell-mediated degradation of heparan sulfate proteoglycans (HSPG) in basement membranes (BM) or extracellular matrix (ECM) has been demonstrated.<sup>1–3</sup> Cleavage of HS results in disassembly of subendothelial ECM and hence may play a decisive role in extravasation of blood-borne cells. Among the cellular enzymes involved in BM/ECM degradation is an endo- $\beta$ -D-glucuronidase called heparanase that cleaves HS at specific intrachain sites.<sup>1,3–5</sup> Expression of heparanase correlates with the metastatic potential of tumor cells<sup>4,6–8</sup> and with the ability of activated cells of the immune system to leave the circulation and elicit both inflammatory and autoimmune responses.<sup>3,6</sup> Elevated heparanase levels have been detected in metastatic cancer cells, in sera from metastatic tumor-bearing animals, in tumor biopsies of cancer patients and in the urine of patients with aggressive metastatic disease.<sup>1,6</sup> Although these phenomena are well documented, it has taken 25 years to isolate the heparanase gene. Recently, several groups have concomitantly reported the successful isolation and cloning of human heparanase as the first cloning example of a mammalian HS-degradative enzyme.<sup>9–12</sup> The isolated cDNA sequences derived from normal (human placenta, platelets) or metastatic cells represent the same gene (*hpa-1*). A gene homologue to *hpa-1*, named *hpa-2*, has also been reported.<sup>13</sup> Alternative splicing of the *hpa-2* transcript yielded 3 different mRNAs (*hpa-2a,b,c*),

encoding putative proteins of 480, 534 and 592 amino acids, respectively. However, these proteins have neither been isolated nor functionally studied for heparanase-like enzymatic activity.<sup>13</sup>

Angiogenesis is critical for the growth of solid tumors and development of metastases. A number of factors have been linked to the angiogenic response including proangiogenic cytokines, angiogenesis modulators and growth factors.<sup>14,15</sup> Of importance, ECM HSPG are directly involved in angiogenesis by serving as a reservoir for potent angiogenic factors (basic fibroblast growth factor [bFGF], vascular endothelial growth factor [VEGF] and others) that can be extracted from ECM produced *in vitro*.<sup>16,17</sup> Therefore, heparanase can be relevant in both invasive (ECM HSPG degradation) and angiogenic (angiogenic factors mobilization from ECM HSPG) events.

Several chemotherapeutic drugs have been analyzed for their potential as antiangiogenic agents including suramin, a polysulfonated naphthylurea. Suramin has been studied as an antineoplastic agent for adrenal cortical malignancies, prostate carcinoma and lymphoma.<sup>18,19</sup> Although the exact mechanism of suramin's antineoplastic activity is not known, it has been shown to nonspecifically inhibit the binding of a number of growth factors and interfere with the glycosaminoglycan catabolism, which is associated with cell proliferation.<sup>20,21</sup> Suramin has also been shown to inhibit angiogenesis and heparanase by independent groups.<sup>22,23</sup>

The clinical use of suramin has been limited by its toxicity. Consequently, suramin analogues have been developed with equivalent or better antitumor activity and less toxicity.<sup>24,25</sup> A number of suramin analogues have been synthesized possessing antiproliferative effects on tumor cells *in vitro* and angiostatic effects on embryologic angiogenesis *in vivo*.<sup>24,26</sup>

We investigated the antiheparanase and antiheparanase-angiogenic effects of a selected group of newly developed suramin analogues and potent angiogenic inhibitors: NF 127, NF 145 and NF 171. Here we demonstrate that these suramin analogues (i) inhibit heparanase-mediated degradation of subendothelial ECM; (ii) inhibit heparanase itself; (iii) possess higher heparanase inhibitory activities than suramin. Of equal importance, we report that these compounds are able to inhibit angiogenic events through heparanase-mediated modalities.

### MATERIAL AND METHODS

#### Chemicals

Suramin and suramin analogues were kindly provided by Drs. Antonio Gagliardi and Delwood Collins (Departments of Obstetrics/Gynecology and Physiology, University of Kentucky, Lexington).

\*Correspondence to: Dr. Dario Marchetti, Department of Comparative Biomedical Sciences, Room 2522, School of Veterinary Medicine, Louisiana State University, Skip Bertman Drive, Baton Rouge, LA 70803, USA. Fax: +225-578-9895. E-mail: dmarchetti@vetmed.lsu.edu

Received 17 July 2002; Revised 7 October 2002; Accepted 15 November 2002

ton, KY). The analogues were generated in the laboratory of Dr. Peter Nickel and coworkers (Pharmaceutical Institute, University of Bonn, Bonn, Germany).<sup>24</sup> Their molecular weight, chemical structure and antiangiogenic properties have been reported.<sup>25–27</sup>

#### Cells and tissue culture

Early passage human brain-metastatic melanoma cells (70W line) were maintained as monolayer cultures in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient medium (DMEM/F-12; GIBCO, Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT).<sup>7</sup> Human 70W cells were chosen as a source of heparanase because they are highly invasive and produce the enzyme at elevated levels vs. parental MeWo.<sup>7,28</sup> Murine brain endothelial (MBE) cells were grown in dishes coated with gelatin (0.5%) and containing DMEM/F12 plus 5% FBS and 50 µg/ml endothelial cell growth factor (ECGF; Biomedical Technologies, Stoughton, MA) reconstituted in DMEM/F-12/1% FBS.<sup>29</sup> Upon reaching confluency, the cells were split with trypsin (0.25%)-EDTA at an optimal ratio of 1:4. Human RL95 cells were grown in 1:1 (v/v) DMEM/F12 supplemented with 10% FBS (v/v).<sup>28</sup> The RL95 cell line, an adenocarcinoma of the uterus, was chosen as a source of HS because of the specific heparanase degradation of cell-associated HS from this cell line and because > 95% of the glycosaminoglycans (GAG) chains made by these cells is HS.<sup>28–30</sup> The [<sup>35</sup>S]HS labeling required the use of a special medium that was sulfate depleted (RPMI 1640, Invitrogen Life Technologies, Carlsbad, CA). Cell lines were periodically checked for *Mycoplasma* contamination (Geneprobe kit; San Diego, CA) and only *Mycoplasma*-free cell lines were used in our study.

#### Cell growth analysis

A fluorescence cell viability assay was used for the cell growth studies. Briefly, 70W cells ( $1 \times 10^4$ ) were seeded in each well of 48-multiwell tissue culture plates and cultured in 0.5 ml of DMEM/F-12 plus 5 µg/ml insulin, 5 µg/ml transferrin and 25 nM sodium selenite. After incubation in absence or presence (up to 200 µM) of suramin or NF 127, NF 145 and NF 171, respectively, cells were rinsed with 1,000 volumes of tissue-culture-grade Dulbecco's PBS without  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$  or pH indicator (DPBS) and exposed to 1.17 µM calcein-AM (Molecular Probes, Eugene, OR) for 45 min at 37°C. After sequential washes, plates were scanned using a Cytofluor 4000 (Applied Biosystems, Foster City, CA) with emission filters to detect calcein fluorescence.<sup>7</sup> Cell numbers were also determined by using a cell counter (Z1 counter, Beckman Coulter, Miami, FL) to obtain an optimal ratio between fluorescence and cell number.

#### Subendothelial matrix degradation

Preconfluent cultures of cloned endothelial cells were plated into 4-well plates at an initial density of  $2.0 \times 10^5$  cells/ml and cultured in sulfate-free Fisher medium plus 4% dextran T-40 for 12 days.  $\text{Na}_2[^{35}\text{S}]\text{SO}_4$  was then incubated at 50 µCi/ml for 72 hr on day 5 after seeding without medium change. The subendothelial ECM was exposed by dissolving the cell layer for 5 min at 25°C with PBS containing 0.5% Triton X-100 and 20 mM  $\text{NH}_4\text{OH}$ , then washed 4 times with PBS.<sup>29</sup> Melanoma cells (70W) were harvested from subconfluent cultures and resuspended in DMEM/F-12 containing 5% FBS. Cell suspensions ( $5 \times 10^5$  cells/ml) and 100 µl aliquots of DMEM/F-12 containing either suramin or NF 127, NF 145 and NF 171 were placed at various concentrations on isolated ECM and incubated for 24–72 hr at 37°C in a  $\text{CO}_2$  incubator. Culture supernatants were withdrawn and centrifuged at 18,000g for 10 min. The [<sup>35</sup>S]sulfate radioactivity released in the supernatant was analyzed by gel filtration on a Sepharose CL-6B column ( $0.9 \times 30$  cm), as previously described.<sup>3,29,30</sup> Radioactivity was quantified on a liquid scintillation counter. Each experiment was performed 3 times, and the variation of elution positions ( $K_{av}$  values) did not exceed  $\pm 15\%$ . Controls to demonstrate heparanase-type action (heparanase cleaves  $\beta$ -glucuronosyl linkages at

HS intra-chain sites) were also performed. HS degradation products were tested for their susceptibility to heparinase, chondroitinases ABC/AC and *Flavobacterium* heparitinases digestion (Seikagaku America, St. Petersburg, FL).<sup>30</sup>

#### Preparation of heparanase-containing cellular extracts and heparanase purification

Subconfluent 70W cells ( $2 \times 10^8$ ) were harvested and solubilized in 50 mM Tris-HCl, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Company, St. Louis, MO), 5 mM *N*-ethylmaleimide (Sigma), 0.05% (w/v) sodium azide, 0.5% (w/v) Triton X-100 at 4°C for 30 min. The cell extract was centrifuged at 12,000g for 30 min at 4°C, and protein concentration was determined using the BCA protein assay protocol (Pierce, Rockford, IL). The cellular extract (20–70 µg of protein) was then incubated in specific heparanase assays employing radiolabeled HS substrates as described below.

Heparanase was purified as reported.<sup>9</sup> Cellular extracts from 70W cells were loaded onto a heparin-Sepharose column ( $5.0 \times 10$  cm), washed in buffer containing 0.5 M NaCl and eluted in column buffer containing 1.0 M NaCl. The eluate was then diluted with an equal volume of dilution buffer 1 (50 mM sodium acetate, pH 6.0, 0.4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid or CHAPS). The heparin-Sepharose eluted fractions were applied to a ConA-agarose column ( $2.5 \times 10$  cm) at a flow rate of 1 ml/min. The column was washed with ConA buffer (50 mM sodium acetate, pH 6.0, 0.5 M NaCl, 0.2% CHAPS), and heparanase was eluted with ConA buffer containing 0.7 M  $\alpha$ -methylmannose. The eluate was concentrated and diluted with 5 volumes of dilution buffer 2 (50 mM sodium acetate, pH 6.0, 0.2% CHAPS), and ConA-agarose eluted fractions were applied to a carboxymethyl-Sepharose column ( $2.5 \times 10$  cm) at a flow rate of 1 ml/min. The column was washed with buffer (50 mM sodium acetate, pH 6.5, 0.1–0.5 M NaCl, 0.2% CHAPS) and eluted with a step gradient of NaCl from 0.1–0.5 M. Fractions containing heparanase were collected, concentrated and mixed with an equal volume of 5 M NaCl solution. The purified fractions from carboxymethyl-Sepharose were applied to a phenyl-Sepharose column ( $2.5 \times 10$  cm) equilibrated with phenyl buffer A (50 mM Tris-HCl, 2.5 M NaCl, pH 7.3) and washed at a flow rate of 0.5 ml/min from 0–40 min. The heparanase was eluted with a gradient of phenyl buffer B (50 mM Tris-HCl, 0.1% CHAPS, pH 7.3) from 0–100% over 100 min. The heparanase-containing fractions were collected, concentrated and purity was visualized by silver-stained SDS-PAGE. The concentration of protein was measured at various purification steps by the BCA protein assay (Pierce).

#### Heparanase assay

Heparanase was assayed as previously described<sup>7,28,30</sup> with some modifications. Heparanase activity was determined by degradation of [<sup>35</sup>S]HS using high-speed gel permeation column chromatography (HPLC).<sup>7,28</sup> The heparan sulfate (HS) used was purified from the human cell line RL95. The purification, [<sup>35</sup>S] sulfate radiolabeling, and use of cell-associated HS subpopulations from RL95 cells has been previously reported.<sup>28</sup> The molecular weight of the specific HS subpopulations had a median value of 70,000 kDa as determined by agarose gel electrophoresis and Superose 6 chromatographic analysis using GAG chains of defined molecular weight size as calibrating standards.<sup>28,29</sup>

Cellular extracts or purified heparanase from 70W cells were incubated at 37°C with [<sup>35</sup>S]HS in 100 µl of 50 mM sodium acetate at pH 4.2. Reaction was terminated by adding 100 µg of heparin and by heating samples for 5 min at 100°C. The products of [<sup>35</sup>S]HS yielded by heparanase reaction were analyzed by HPLC analysis. Aliquots of the reaction were injected into a TSK gel G3000 PWX2 column (7.8 mm  $\times$  30 cm; 6 µm particle size; Toso Haas, Montgomeryville, PA) and run at 0.5 ml/min. Eluents were collected and counted in a liquid scintillation counter. Heparanase activity was determined by measuring the decrease of

radioactivity in the first one-half area of intact [ $^{35}\text{S}$ ]HS peak chromatogram.

#### *Chemoinvasion assay*

The invasive properties of melanoma 70W cells were assayed by Transwell cell culture chambers and fluorescence plate scanner analysis.<sup>7</sup> Briefly, purified heparan sulfate proteoglycan (HSPG) was applied to the filters (0.5 mg/ml). Alternatively, reconstituted basement membrane (Matrigel<sup>TM</sup>; BD Biosciences, Discovery Labware, Bedford, MA) was diluted 1:30 in cold DMEM/F12 without phenol red and applied onto the upper surface of Transwell (6.5 mm diameter, 8  $\mu\text{m}$  pore size) polycarbonate filter inserts (Costar, Cambridge, MA). The lower chamber contained a mixture of 0.5% low-gelling agarose (FMC Bio Products, Rockland, ME), 0.5% gelatin (Sigma) with addition of 10 nM of the chemotactic peptide N-formyl-met-leu-phe (Sigma) and 25% phenol-red-free fresh murine brain endothelial cell-conditioned medium (MBE-CM, 48 hr).<sup>7</sup> Cells suspended in serum-free, phenol-red-free DMEM/F-12 were seeded into the upper compartment at  $2.0 \times 10^4$  cells/filter. After 24 hr incubation at 37°C, the upper chambers were carefully removed and the bottom chambers were treated with 1  $\mu\text{M}$  calcein-AM for 1 hr at 37°C. After 4 washes with PBS, the plates were analyzed for fluorescence using a computerized fluorescence plate scanner system (Cytofluor 4000, Applied Biosystems, Foster City, CA), and the formation of calcein was monitored by measuring the increase in fluorescence at 530 nm (BB4 settings of Cytofluor).

#### *Fluorometric microculture cytotoxicity assay*

A fluorometric microculture cytotoxicity assay (FMCA) procedure was used to study the cytotoxicity of suramin-analogue compounds. The FMCA is based on the measurement of fluorescence generated from the hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes and has been previously described in detail.<sup>31</sup> Briefly, 70W cells were resuspended in complete medium, and 180  $\mu\text{l}$  of cell suspension was seeded into the wells of 96-well experimental microtitre plates. The densities of the 70W cells were  $5.0 \times 10^3$ – $2.0 \times 10^4$  cells/well. Suramin and its analogues were tested in triplicate and at 5 different concentrations. Wells with cells but without compounds served as controls and wells with only culture medium served as blanks. The plates were subsequently incubated at 37°C in a humidified atmosphere containing 5%  $\text{CO}_2$  for 72 hr. At the end of the incubation period, the plates were centrifuged (800 g, 5 min) and the medium was removed by aspiration. After 1 wash in PBS, 100  $\mu\text{l}$ /well of FDA (10  $\mu\text{g}/\text{ml}$ ) dissolved in physiologic buffer was added. The plates were then incubated for 30 min and the generated fluorescence from each well was measured at 538 nm in a 96-well scanning fluorometer. The fluorescence signal was found to be proportional to the number of intact cells in the well. Quality criteria for a successful analysis included a fluorescence signal in the control wells of more than 5 $\times$  the mean blank value and a coefficient of variation in the control wells of < 30%. A cell survival index (SI) was subsequently derived and defined as the fluorescence in the experimental wells compared to that in control wells, with the blank values subtracted.  $\text{IC}_{50}$  values were determined as the concentration of compound giving an SI of 70%.

#### *Encapsulation of bFGF-HSPG in alginate microspheres and in vivo angiogenic assays*

A method for the preparation and encapsulation of HSPG-bFGF in alginate microspheres was used as described elsewhere.<sup>32</sup> Briefly, HSPG-bFGF exposed to heparanase in presence or absence of suramin analogues was mixed with alginate gel (1.8%). The mixture was used to prepare beads, at a volume of 10 ml, by dropping them into a hardening solution of 1.5%  $\text{CaCl}_2$ . After polymerization, the alginate beads were extensively washed with saline containing  $\text{CaCl}_2$  and stored at 4°C or used immediately. The incorporation and release kinetics of compounds from alginate beads were determined.<sup>33</sup> In addition, alginate beads containing

the separate constituents (bFGF, HSPG-bFGF, suramin and heparanase) were prepared and tested in rabbit ear chambers.

Alginate beads were implanted under general anesthesia (70 mg/kg ketamine and 10 mg/kg xylazine) in the ears of female New Zealand white rabbits. For observations, the animals were sedated by butorphanol and acepromazine (each at 1 mg/kg). The surgical procedure and postoperative care was as previously described.<sup>34</sup> A low-power stereo microscope with a stage adjustment for viewing the chambers was used for taking microphotographs at weekly intervals. An angiogenic score was subsequently obtained by triple-blind evaluation of the microphotographs taken after analysis of the chambers at weekly intervals. The following 4 parameters were used: (i) the total area of the chamber covered by vessels; (ii) the number of vessels originating from openings in the chambers; (iii) the length of large anastomosing vessels; (iv) the number of anastomosing vessels. The maximum numerical value for each parameter was 10, adding to a maximal angiogenic score of 40 as arbitrary units (AU).

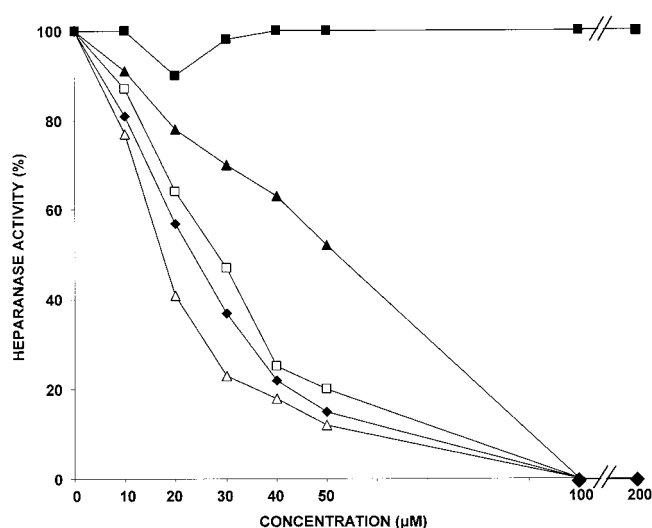
## RESULTS

#### *Effects of suramin analogues NF 127, NF 145 and NF 171 on heparanase activity*

The purpose of our study was to analyze the effectiveness of selected suramin analogues named NF 127, NF 145 and NF 171 to inhibit heparanase activity and to relate it to suramin, a known heparanase inhibitor.<sup>23</sup> These compounds are naphthalenetrisulfonic acid derivatives of suramin and belong to a large category of suramin analogues whose chemical structure, molecular weight, and, more importantly, their capability to act as potent antiangiogenic agents have been determined and reported.<sup>25–27</sup> The ability of 70W cells to degrade HS in the ECM was studied by allowing the cells to interact with naturally produced sulfate-labeled ECM followed by gel filtration analysis of degradation products released into a culture medium.<sup>3,30</sup> Degradation of HS in the ECM was studied by incubation (72 hr at 37°C, pH 6.7) of high-heparanase-containing 70W previously exposed to suramin or, alternatively, to NF 127, NF 145 and NF 171 with a metabolically sulfate-labeled ECM produced by cultured endothelial cells (MBE). Sulfate-labeled degradation products released into the incubation medium were subjected to gel filtration on Sepharose-6B columns.<sup>3,30</sup> In the presence of the incubation medium alone, there was a constant spontaneous release of labeled material consisting almost exclusively (> 90%) of high-molecular-weight fragments eluted at or next to  $V_0$  of the column.<sup>30</sup> The proteolytic activity residing in the ECM itself is responsible for the release of the high-molecular-weight material. This nearly intact HSPG provides a soluble substrate for subsequent degradation by heparanase.

Accumulation in the medium of low-molecular-weight, sulfate-labeled degradation fragments was inhibited in the presence of suramin analogues NF 127, NF 145 and NF 171 but not in their absence (Fig. 1). Controls using suramin were performed because melanoma heparanase is noncompetitively inhibited by suramin.<sup>23</sup> That these HS degradative products were heparanase-digested was confirmed by several control experiments demonstrating that they were (i) 3–5-fold smaller than intact HS side chains;<sup>1,4,9</sup> (ii) released from the ECM by treatment with alkaline borohydride;<sup>30</sup> and (iii) resistant to further digestion with chondroitinase ABC but susceptible to deamination by nitrous acid.<sup>30</sup>

In a second set of experiments, heparanase purified from human 70W cells was incubated (24 hr at 37°C, pH 6.0) with sulfate-labeled ECM in the absence or presence of increasing concentrations of either suramin or NF 127, NF 145 and NF 171. Their addition resulted in a corresponding decrease of heparanase activity to background levels at 100  $\mu\text{M}$  concentrations of compounds.  $\text{IC}_{50}$  values for these analogues varied from 18–28  $\mu\text{M}$ , which was consistently lower than the one observed for suramin (42  $\mu\text{M}$ ; Fig. 2). Also, we performed heparanase assays by HPLC chromatographic analysis of radiolabeled HS degradation products. When radiolabeled HS was analyzed by HPLC, increased heparanase



**FIGURE 1** – Suramin analogues NF 127, NF 145 and NF 171 inhibit heparanase activity expressed by human 70W melanoma cells. Human 70W melanoma cells ( $1 \times 10^6/35$  mm dish) were incubated (72 hr at  $37^\circ\text{C}$ , pH 6.7) on top of sulfate-labeled ECM, in absence (filled squares) or presence of increasing concentrations of suramin (filled triangles), NF 127 (open squares), NF 171 (filled diamonds) and NF 145 (open triangles). Sulfate-labeled degradation products released into the incubation medium were analyzed by gel filtration on Sepharose-6B column chromatography. Heparanase activity was expressed as  $K_{av} \times$  total cpm eluted in the peak (fractions 35–45) of the column.<sup>30</sup> One hundred percent values ranged from  $2.7$ – $3.0 \times 10^4$  cpm. Each experiment was performed 3 times, and the variation of elution positions ( $K_{av}$  values) did not exceed  $\pm 15\%$ .

activity corresponded to the amount of 70W heparanase present in the assay (data not shown). Conversely, in experiments where NF 127, NF 145 and NF 171 (or control suramin) were incubated with purified heparanase, inhibition of its activity by specific heparanase assays was observed (Table I).

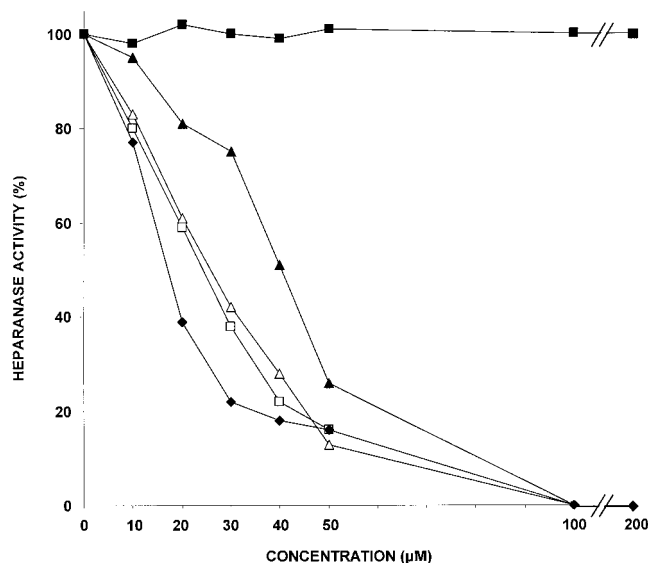
Finally, the effects of suramin, NF 127, NF 145 and NF 171 on 70W cell growth viability were tested. In serum-free culture medium supplemented with insulin and transferrin, the growth of 70W melanoma cells was not significantly affected by suramin or related compounds at  $100 \mu\text{M}$  (Fig. 3) or at higher concentrations of compounds (up to  $200 \mu\text{M}$ , data not shown).

#### *Inhibition of melanoma cell invasion by suramin analogues NF 127, NF 145 and NF 171*

The effect of NF 127, NF 145 and NF 171 on the *in vitro* invasive capacity of brain-metastatic, high-heparanase-producing 70W cells was tested by seeding cells onto purified HSPG-coated filters. Invasion assays were then performed through HSPG-coated filters to determine whether heparanase production in 70W cells could be blocked by these suramin analogues. The 70W cells were treated with different concentrations of NF 127, NF 145 and NF 171, seeded on HSPG-coated filters and allowed to invade for 24 hr at  $37^\circ\text{C}$ . The results from 4 independent experiments indicated that the invasion process was effectively inhibited by NF 127, NF 145, and NF 171 at  $10 \mu\text{M}$  (and higher) concentrations with a significant decrease of invasive values found at a concentration of compounds as low as  $1.5 \mu\text{M}$ . The results from these experiments are shown in Table II.

In a second sets of experiments, invasion assays were performed using the more commonly applied Matrigel<sup>TM</sup> as a coating barrier on Transwell filters. Results from these experiments are included for comparative purposes and confirmed inhibition of the invasion process by suramin and the 3 suramin analogues tested (Table III).

Thirdly, a semiautomated *in vitro* fluorometric microculture cytotoxicity assay (FMCA) was employed to evaluate the cytotox-



**FIGURE 2** – Inhibition of purified 70W heparanase by NF 127, NF 145 and NF 171. Sulfate-labeled ECM was incubated (24 hr at  $37^\circ\text{C}$ , pH 6.0) with purified preparations of human 70W heparanase ( $3.0 \mu\text{g}/\text{ml}$ ) in absence (filled squares) or in presence of increasing concentrations of NF 127 (open triangles), NF 171 (open squares), NF 145 (filled diamonds) and suramin (filled triangles). Sulfate-labeled degradation products released into the incubation medium were analyzed by gel filtration on Sepharose-6B column chromatography. Heparanase activity is expressed as  $K_{av} \times$  cpm eluted in the peak of the column.<sup>30</sup> Three independent experiments were performed confirming the results shown.

**TABLE I** – INHIBITION OF 70W HEPARANASE ACTIVITY BY SURAMIN ANALOGUES<sup>1</sup>

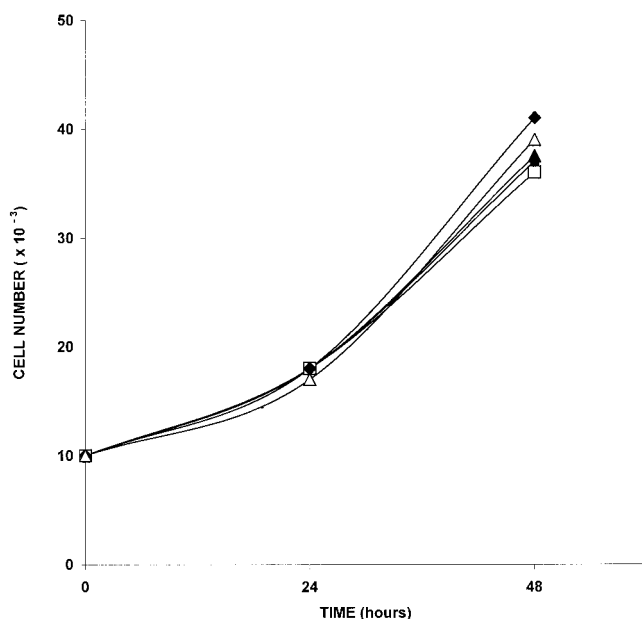
Compound	Heparan sulfate degradation activity (Mean cpm $\pm$ SD)
None added	$13,409 \pm 420$
+ Suramin	$6,978 \pm 342^*$
+ NF 127	$4,696 \pm 438^*$
+ NF 145	$3,828 \pm 705^*$
+ NF 171	$4,397 \pm 381^*$

<sup>1</sup>Purified heparanase was preincubated with suramin, NF 127, NF 145 and NF 171 ( $30 \mu\text{M}$  for 24 hr at  $37^\circ\text{C}$ ) then analyzed by specific heparanase assays (see Material and Methods). Data shown represent subtraction of control values (absence of heparanase in [ $^{35}\text{S}$ ]HSA assay) from data of test samples.  $^*p < 0.01$  for 4 separate experiments.

icity of the 3 suramin analogues using human 70W cells. In these cells, NF 127, NF 145 and NF 171 exhibited comparable activities, as mean  $\text{IC}_{30}$  values, to each other and to suramin (Table IV).

#### *Inhibition of heparanase-induced angiogenesis by suramin analogues NF 127, NF 145 and NF 171*

Since the abilities of bFGF to bind ECM HSPG and to act as a potent angiogenic factor have long been recognized,<sup>14,16</sup> we have evaluated the ability of NF 127, NF 145 and NF 171 to modulate angiogenic heparanase activity *in vivo* by using the rabbit ear chamber method. This *in vivo* angiogenic assay has been used for the past 50 years as a well-controlled *in vivo* model to study angiogenesis, wound healing and tissue responses.<sup>34</sup> bFGF bound to HSPG and encapsulated in alginate gel acts as a controlled release delivery system.<sup>33</sup> The encapsulation efficiency of alginate microspheres was studied by using radioiodinated bFGF ([ $^{125}\text{I}$ ]bFGF) and comparing the amount of encapsulated bFGF with that initially present in solution. [ $^{125}\text{I}$ ]bFGF release kinetics were subsequently determined. A cumulative bFGF release into  $0.15\%$  saline at  $37^\circ\text{C}$  was noted over time, reaching  $15$ – $20\%$  of



**FIGURE 3**—Effects of suramin and suramin analogues on 70W melanoma cell growth. Human 70W cells ( $1 \times 10^4$ ) were seeded in each well of 48-multiwell tissue culture plates and cultured in 0.5 ml of DMEM/F-12 plus 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin and 25 nM sodium selenite in the absence (filled squares) or presence (100  $\mu$ M) of NF 127 (open triangles), NF 171 (open squares), NF 145 (filled diamonds) and suramin (filled triangles). Cell growth was monitored by calcein-AM methodology using a Cytofluor 4000 (see Material and Methods). Background fluorescence levels were determined and subtracted to obtain specific fluorescence. Each point represents the average of triplicate determinations (SD < 5%).

growth factor release at 5–10 days, respectively, and a plateau of 30% at approximately 4 weeks (data not shown). Secondly, control experiments were performed using alginate beads containing only bFGF, HSPG-bFGF, suramin or heparanase, respectively, with subsequent angiogenic score determination. Differential angiogenic scores were obtained among the various constituents with the highest values found in the presence of purified heparanase (Fig. 4).

Implantation of HSPG-bFGF beads previously incubated with purified heparanase, and in presence of suramin analogues, into rabbit ear chambers produced a significantly decreased vascularization. Photomicrographs were evaluated using several parameters and an angiogenic score was obtained of significant value for heparanase-induced angiogenesis in the presence of heparanase (but without suramin or suramin analogues) with inhibition of the enzyme in a dose-dependent manner in their presence. Representative experiments using NF 145, in our hands the most active compound among the ones analyzed, are shown in Figure 5. When heparanase-treated samples were analyzed, fine capillaries and occasionally large vessels penetrated the chamber, covering almost its entire surface (Fig. 5, lane 4). However, in the presence of NF 145, a distinct and significant difference in the degree and pattern of vascularization was observed at NF 145 concentrations of 135  $\mu$ M and 180  $\mu$ M (Fig. 5, lane 3 and 2, respectively) until only a limited capillary ingrowth was found at a 230  $\mu$ M concentration of the drug (Fig. 5, lane 1).

Suramin and suramin analogues NF 127 and NF 171 were similarly tested in rabbit ear chambers invasion assays. They induced a statistically significant inhibition of heparanase-induced angiogenic response (Table V). Using this assay, we found suramin to have an  $IC_{50}$  value of 538  $\mu$ M. All 3 suramin analogues possessed more potent inhibitory properties of heparanase-induced angiogenesis compared to suramin, with

$IC_{50}$  values ranging from 118  $\mu$ M for NF 145 to 181  $\mu$ M for NF 127 (Table V).

## DISCUSSION

In our study, we have demonstrated that the suramin analogues NF 127, NF 145 and NF 171 inhibited heparanase activity as well as heparanase-induced ECM HS degradation and angiogenesis. Specifically, heparanase inhibition by these compounds correlated with decreased invasive capacity of human melanoma 70W cells by *in vitro* invasion assays and decreased blood vessel formation by *in vivo* angiogenic assays, thus linking the antiangiogenic properties of NF 127, NF 145 and NF 171 with their ability to inhibit heparanase.

Angiogenesis is a critical event in tumor cell metastasis because the growth of solid tumors to a significant size depends upon it.<sup>15,35,36</sup> Therefore, angiogenic inhibitors have become an important potential approach for cancer therapy. Heparanase is an HS-specific endo- $\beta$ -D-glucuronidase and a critical molecular determinant of tumor metastasis because its activity directly correlates with metastasis of tumor cells, being involved in their invasive-ness.<sup>1-3,7-10</sup>

We and others have postulated 2 roles of heparanase in melanoma metastasis: One is that heparanase is relevant to the extravasation of melanoma cells into secondary organs by degrading subendothelial ECM HSPG degradation, whereas the second pertains to heparanase's ability to release angiogenic factors, stored within ECM HSPG, at the metastatic site and leading edge of neovascularization. The mobilization of these factors and their angiogenic stimulation is a critical event in angiogenesis.<sup>14-16</sup>

A number of studies have demonstrated that heparanase activity and experimental metastasis are inhibited by nonanticoagulant and low-molecular-weight species of heparin.<sup>2,6,37</sup> It also has been demonstrated that a variety of sulfated polysaccharides other than heparin can inhibit heparanase and tumor growth.<sup>38-40</sup> Finally, and relevant to the purpose of our study, reports have documented heparanase inhibition by suramin.<sup>7,23,40</sup>

Suramin, a polysulfonated naphthylurea, was originally developed as a trypanocidal agent.<sup>18,41</sup> Results have been published indicating that suramin is a useful antitumorigenic agent in human malignancies.<sup>18</sup> However, the clinical use of suramin has been limited by its toxicity. The most serious adverse effect of suramin use is the development of polyneuropathy, which ranges from stocking-glove paresthesias to paralysis.<sup>42,43</sup> To resolve this problem, suramin analogues have been developed with equivalent or better antitumor activity and less toxicity.<sup>24,44</sup>

We hypothesized that some of these analogues could be effective antiheparanase agents. In particular, NF 127, NF 145 and NF 171 were chosen, among others, because they were proven to possess a superior angiogenic inhibitory activity than suramin, and for their structural similarities to suramin being naphthalenetrisulfonic acid derivatives.<sup>25-27</sup> We examined the effect of these compounds on melanoma cell heparanase and demonstrated that NF 127, NF 145 and NF 171 are more potent heparanase inhibitors than suramin itself.

The mechanism(s) underlying antitumorigenic effects of suramin and its analogues are poorly understood. It has been postulated that they bind to growth factors, thus altering the binding ability of growth factors to their respective receptors.<sup>20,21,44</sup> The enzymes involved in the catabolism of glycans, such as ganglioside neuraminidase,  $\beta$ -glucuronidase, hyaluronidase and iduronate sulfatase, have also been found to be inhibited by suramin, perhaps accounting for the aberrant accumulation of sialogangliosides and glycosaminoglycans, including HS, dermatan sulfate and hyaluronic acid, in various organs of suramin-treated patients and animals.<sup>18</sup> Certainly, because heparanase is the major endoglycosidase initiating the sequential cleavage of HS linked to ECM HSPG, its inhibition is much more critical in tumor metastasis than the inhibition of exoglycosidases and/or sulfatases.

TABLE II – INHIBITION OF 70W MELANOMA CELL INVASION BY SURAMIN ANALOGUES: HSPG AS COATING BARRIER

Concentration ( $\mu$ M)	Suramin	NF 127	NF 145	NF 171
0	13.07 $\pm$ 0.01 <sup>1</sup>	14.03 $\pm$ 0.02	12.91 $\pm$ 0.15	13.02 $\pm$ 0.02
10	12.15 $\pm$ 0.03**	11.36 $\pm$ 0.03*	9.11 $\pm$ 0.06**	9.89 $\pm$ 0.02*
25	9.67 $\pm$ 0.18**	8.69 $\pm$ 0.06**	5.84 $\pm$ 0.02**	3.51 $\pm$ 0.02**
50	7.71 $\pm$ 0.01**	2.81 $\pm$ 0.01**	2.51 $\pm$ 0.04***	1.95 $\pm$ 0.02**
100	0	0	0	0
200	0	0	0	0
<i>n</i> = 3 <sup>2</sup>				

<sup>1</sup>70W arbitrary fluorescence units (AFU) at the end of incubation period (24 hr). AFU at time 0 were considered equal to 1.00.<sup>-2</sup>*n*, data displayed are means of 3 independent experiments with quadruplicate assays performed in each experiment. \**p* < 0.005, \*\**p* < 0.07, \*\*\**p* < 0.001.

TABLE III – INHIBITION OF 70W MELANOMA CELL INVASION BY SURAMIN ANALOGUES: MATRIGEL™ AS COATING BARRIER

Concentration ( $\mu$ M)	Suramin	NF 127	NF 145	NF 171
0	9.41 $\pm$ 0.01 <sup>1</sup>	10.38 $\pm$ 0.04	8.77 $\pm$ 0.03	9.37 $\pm$ 0.30
10	8.26 $\pm$ 0.02**	7.38 $\pm$ 0.01*	6.83 $\pm$ 0.05**	7.71 $\pm$ 0.04**
25	6.77 $\pm$ 0.03**	6.51 $\pm$ 0.02**	4.14 $\pm$ 0.01***	2.42 $\pm$ 0.03*
50	6.55 $\pm$ 0.02*	4.49 $\pm$ 0.03**	2.93 $\pm$ 0.03*	2.08 $\pm$ 0.01**
100	0	0	0	0
200	0	0	0	0
<i>n</i> = 3 <sup>2</sup>				

<sup>1</sup>70W arbitrary fluorescence units (AFU) at the end of incubation period (24 hr). AFU at time 0 were considered equal to 1.00.<sup>-2</sup>*n*, data displayed are means of 3 independent experiments with quadruplicate assays performed in each experiment. \**p* < 0.005, \*\**p* < 0.05, \*\*\**p* < 0.001.

TABLE IV – ACTIVITY OF SURAMIN ANALOGUES IN HUMAN 70W CELLS

Compound	IC <sub>30</sub> (nmol/ml) <sup>1</sup>
Suramin	364
NF 127	362
NF 145	374
NF 171	334

<sup>1</sup>IC<sub>30</sub> denotes compound concentration resulting in a survival index of 70%. Shown are mean values of 3 independent experiments.

We have considered that an alternative (or complementary) mechanism of action of suramin analogues as anticancer agents in general and antiangiogenic agents in particular is the inhibition of ECM degradative enzymes like heparanase. The IC<sub>50</sub> values for NF 127, NF 145 and NF 171 were approximately one-third to one-fourth of those seen for suramin, being valid for both inhibition of the enzyme and its angiogenic-related effects. Other enzymes and/or factors may be responsible for the antiangiogenic properties of these compounds, whose inhibition capabilities and mode of action may complement the ones seen for heparanase.

A trend that is emerging from studies involving suramin-like compounds is that apart from sulfation or other negatively charged moieties, there are no defined structural requirements for the inhibition of heparanase activity. In general, this may explain why diverse polyanionic molecules such as sulfated polysaccharides,<sup>40</sup> phosphorothioate oligodeoxynucleotides,<sup>45</sup> other synthetic polycarboxylic aromatic compounds,<sup>46</sup> suramin<sup>23</sup> or some of its analogues as reported here all inhibit heparanase despite their different molecular structure and chemical nature. The inhibition by such unrelated polyanionic compounds indicates that both natural polysaccharides and synthetic polyanions may be applied as inhibitors of tumor metastasis. However, it should be taken into account that (i) not every polyanionic molecule is a good heparanase inhibitor *in vitro*; (ii) not all heparanase-inhibiting molecules will efficiently inhibit *in vivo* tumor cell invasion and angiogenesis; and (iii) a number of small molecule inhibitors are currently being developed that do not necessarily fit into the category of polyanionic compounds. Certainly, the efficacy of a given molecule as an effective *in vivo* inhibitor will have to depend on its pharmacokinetic

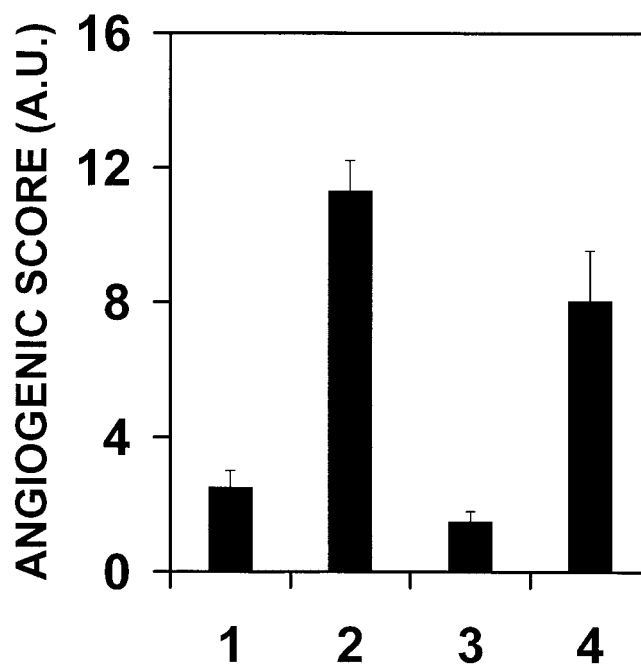
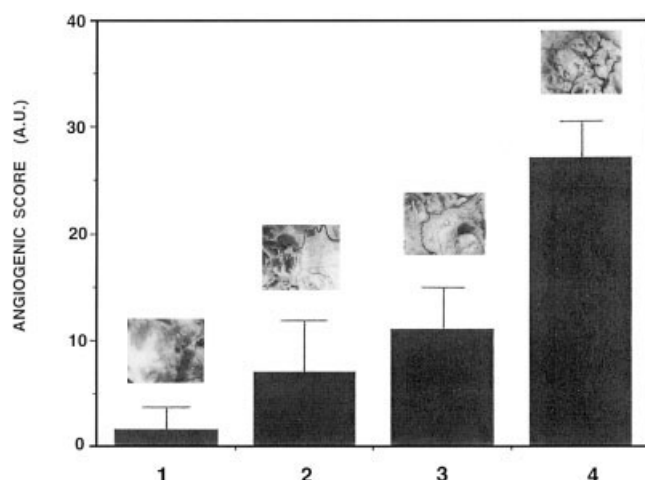


FIGURE 4 – Angiogenic scores of heparanase-induced angiogenesis. Alginate beads containing encapsulated bFGF (lane 1), HSPG-bFGF (lane 2), suramin (lane 3) or purified heparanase (lane 4) were introduced into chambers and implanted in rabbit ears. At 35–45 days post-implantation, the chambers were analyzed by light microscopy. Photomicrographs (6–8 analyzed for each experiment) were evaluated using 4 parameters (see Material and Methods). The maximum numerical value for each parameter is 10, adding to a maximal angiogenic score of 40 as arbitrary units (AU). Concentration of each compound was approximately 40 ng/bead.



**FIGURE 5** – Angiogenesis inhibition score by suramin analogue NF 145. Rabbit ear chambers filled with bFGF HSPG-treated heparanase in the presence of 230  $\mu$ M (lane 1), 180  $\mu$ M (lane 2) or 135  $\mu$ M of NF 145 (lane 3) or in its absence (lane 4) were quantified for angiogenic inhibition. Photomicrographs (10 analyzed for each experiment with bars representing the standard deviation) from rabbit ear chambers were evaluated using 4 different parameters: total area of the chamber covered by vessels; number of vessels originating from chamber openings; length of large vessels; and number of anastomosing vessels. The maximum numerical value for each parameter was 10, adding to a maximal angiogenic score of 40 as arbitrary units (AU).

**TABLE V** – INHIBITION OF HEPARANASE-INDUCED ANGIOGENESIS BY SURAMIN AND SURAMIN ANALOGUES

Compound	IC <sub>50</sub> (nmol/ml) <sup>1</sup>
Suramin	538
NF 127	181
NF 145	118
NF 171	136

<sup>1</sup>Dose of compound that induced 50% inhibition of angiogenesis in the rabbit ear chamber *in vivo* angiogenic model.

properties (*i.e.*, half-life in the circulation, mode of clearance) and potential side effects.

In conclusion, we have demonstrated that 3 suramin analogues, NF 127, NF 145 and NF 171, inhibit human heparanase and heparanase-mediated angiogenesis. We believe that a part of the antineoplastic effect of these drugs may be due to their heparanase inhibitory action. Therefore, we postulate that heparanase can be an important target (or one of the targets) of suramin analogues involved in their mode of action in angiogenesis. Biochemical pathways responsible for their antineoplastic and antiangiogenic effects and the role of heparanase in these pathways will have to be clarified. Our current findings support further investigations of the possible usefulness of these and other suramin analogues, *i.e.*, possessing different structural properties, as heparanase inhibitors and therapeutic agents for metastatic disease.

#### ACKNOWLEDGEMENTS

This work was supported by N.C.I. grant RO1-CA86832-02 to D.M.

#### REFERENCES

- Nakajima M, Irimura T, Nicolson GL. Heparanase and tumor metastasis. *J Cell Biochem* 1988;36:157–67.
- Parish CR, Coombe DR, Jakobsen KB, Bennet FA, Underwood PA. Evidence that sulfated polysaccharides inhibit tumour metastasis by blocking tumour-cell-derived heparanases. *Int J Cancer* 1987;40:511–58.
- Vlodavsky I, Fuks Z, Bar-Ner M, Ariav Y, Schirrmacher V. Lymphoma-cell-mediated degradation of sulfated proteoglycans in the sub-endothelial extracellular matrix: relationship to tumor-cell metastasis. *Cancer Res* 1983;43:2704–11.
- Nakajima M, Irimura T, Di Ferrante N, Nicolson GL. Metastatic melanoma cell heparanase: characterization of heparan sulfate degradation fragments produced by B16 melanoma endoglucuronidase. *J Biol Chem* 1984;259:2283–90.
- Sandback-Pikas D, Li JP, Vlodavsky I, Lindahl U. Substrate specificity of heparanases from human hepatoma and platelets. *J Biol Chem* 1988;263:18770–7.
- Vlodavsky I, Mohsen M, Lider O, Ishai-Michaeli R, Ekre HP, Svahn CM. Inhibition of tumor metastasis by heparanase-inhibiting-species of heparin. *Invas Metast* 1995;14:290–302.
- Marchetti D, Menter D, Jin L, Nakajima M, Nicolson GL. Nerve growth factor effects on human and mouse melanoma cell invasion and heparanase production. *Int J Cancer* 1993;55:692–9.
- Walch ET, Marchetti D. Role of neurotrophins and neurotrophin receptors in the *in vitro* invasion and heparanase production of human prostate cancer cells. *Clin Exp Met* 1999;17:307–14.
- Toyoshima M, Nakajima M. Human heparanase: purification, characterization, cloning, and expression. *J Biol Chem* 1999;274:24153–60.
- Vlodavsky I, Friedmann Y, Elkin M, Aingorn H, Atzman A, Ishai-Michaeli R, Bitan M, Pappo O, Peretz T, Michal I, Spector L, Pecker I. Mammalian heparanase: a novel gene involved in tumor progression and metastasis. *Nature Med* 1999;5:793–802.
- Hulett MD, Freeman C, Hamdorf BJ, Baker RT, Harris MJ, Parish CR. Cloning of mammalian heparanase, an important enzyme in tumor invasion and metastasis. *Nature Med* 1999;5:803–9.
- Kussie PH, Hulmes JD, Ludwig DL, Patel S, Navarro EC, Seddon AP, Giorgio NA, Bohlen P. Cloning and functional expression of a human heparanase gene. *Biochem Biophys Res Comm* 1999;261:183–7.
- McKenzie E, Tyson K, Stamps A, Smith P, Turner P, Barry R, Hircok M, Patel S, Barry E, Stubberfield C, Terrett J, Page M. Cloning and expression profiling of Hpa2, a novel mammalian heparanase family member. *Biochem Biophys Res Comm* 2000;276:1170–7.
- Folkman J, Klagsbrun M. Angiogenic factors. *Science* 1987;235:442–7.
- Folkman J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 1990;82:4–6.
- Folkman J, Klagsbrun M, Sasse J, Wadinski M, Ingber D, Vlodavsky I. Heparin-binding angiogenic protein-basic fibroblast growth factor is stored within basement membrane. *Am J Pathol* 1988;130:393–400.
- Vlodavsky I. A heparin-binding angiogenic protein, basic fibroblast growth factor, is stored within basement membrane. *Am J Pathol* 1988;130:393–400.
- Stein C, La Rocca R, Thomas R, Mcatee N, Myers C. Suramin: an anticancer drug with a unique mechanism of action. *J Clin Oncol* 1989;7:499–508.
- Myers C, Cooper M, Stein C, La Rocca R, Walther M, Weiss G, Choyke P, Dawson N, Steinberg S, Uhrich M, Cassidy J, Kohler D, et al. Suramin: a novel growth factor antagonist with activity in hormone-refractory metastatic prostate cancer. *J Clin Oncol* 1992;10:881–9.
- Coffey R, Leof E, Shipley G, Moses H. Suramin inhibition of growth factor receptor binding and mitogenicity in ARK-2B cells. *J Cell Physiol* 1987;132:143–7.
- Pesenti E, Sol F, Mongilli N, Grandi M, Spreafico F. Suramin prevents neovascularization and tumor growth through blocking of basic fibroblast growth factor activity. *Br J Cancer* 1992;66:367–72.
- Gagliardi A, Hadd H, Collins D. Inhibition of angiogenesis by suramin. *Cancer Res* 1992;52:5073–5.
- Nakajima M, De Chavigny A, Johnson CE, Hamada J, Stein CA, Nicolson GL. Suramin. A potent inhibitor of melanoma heparanase and invasion. *J Biol Chem* 1991;266:9661–6.
- Firsching A, Nickel P, Mora P, Allolio B. Antiproliferative and angiostatic activity of suramin analogues. *Cancer Res* 1995;55:4957–61.
- Gagliardi AR, Kassack M, Kreimeyer A, Muller G, Nickel P, Collins DC. Antiangiogenic and antiproliferative activity of suramin analogues. *Cancer Chemother Pharmacol* 1998;41:117–24.
- Dhar S, Gullbo J, Csoka K, Eriksson E, Nilsson K, Nickel P, Larson R, Nygren P. Antitumor activity of suramin analogues in human tumour cell lines and primary cultures of tumour cells from patients. *Eur J Cancer* 2000;36:803–9.
- Meyers MO, Gagliardi AR, Flattmann GJ, Su JL, Wang Y, Woltering



- EA. Suramin analogues inhibit human angiogenesis in vitro. *J Surg Res* 2000;91:130–4.
28. Marchetti D, McQuillan DJ, Spohn WC, Carson DD, Nicolson GL. Neurotrophin stimulation of human melanoma cell invasion: selected enhancement of heparanase activity and heparanase degradation of specific heparan sulfate subpopulations. *Cancer Res* 1996;56:2856–63.
29. Marchetti D. Specific degradation of subendothelial matrix proteoglycans by brain-metastatic melanoma and brain endothelial cell heparanases. *J Cell Physiol* 1997;172:334–42.
30. Marchetti D, Liu S, Spohn WC, Carson DD. Heparanase and a synthetic peptide of heparan sulfate-interacting protein recognize common sites on cell surface and extracellular matrix heparan sulfate. *J Biol Chem* 1997;272:15891–7.
31. Larsson R, Kristensen J, Sandberg C, Nygren P. Laboratory determination of chemotherapeutic drug resistance in tumor cells from patients with leukemia using a fluorometric microculture cytotoxicity assay. *Br J Cancer* 1992;50:177–85.
32. Read T, Stensvaag V, Vindenes H, Ulvestad E, Bjerkvig R, Thorsen F. Cells encapsulated in alginate: a potential system for delivery of recombinant proteins to malignant brain tumors. *Int J Dev Neurosci* 1999;17:653–63.
33. Edelman RE, Mathiowitz E, Langer R, Klagsbrun M. Controlled release of bFGF. *Biomaterials* 1991;12:619–25.
34. Howden G, Silver I. The use of rabbit ear chamber technique for the study of dental materials. *Int Endodontic J* 1980;13:3–16.
35. Diaz-Flores L, Gutierrez R, Varela H. Angiogenesis: an update. *Histol Histopathol* 1994;9:807–15.
36. Klagsbrun M. Introduction. In: Rifkin DB, Klagsbrun M, eds. *Angiogenesis. Mechanisms and pathobiology*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1987. 1–7.
37. Irimura T, Nakajima M, Nicolson GL. Chemically modified heparins as inhibitors of heparan-sulfate-specific endo-beta-glucuronidase (heparanase) of metastatic melanoma cells. *Biochemistry* 1986;25:5322–8.
38. Miao H-Q, Elkin M, Aingorn E, Ishai-Michaeli R, Stein CA, Vlodavsky I. Inhibition of heparanase activity and tumor metastasis by laminarin sulfate and synthetic phosphorothioate oligodeoxynucleotides. *Int J Cancer* 1999;83:424–31.
39. HersHKoviz R, Mor F, Miao H-Q, Vlodavsky I, Lider O. Differential effects of polysulfated polysaccharide on experimental encephalomyelitis, proliferation of autoimmune T cells and inhibition of heparanase activity. *J Autoimmun* 1995;8:741–50.
40. Parish CR, Freeman C, Brown KJ, Francis DJ, Cowden WB. Identification of sulfated oligosaccharide-based inhibitors of tumor growth and metastasis using novel in vitro assays for angiogenesis and heparanase activity. *Cancer Res* 1999;59:3433–41.
41. Zaniboni A. Suramin: the discovery of an old anticancer drug. *Med Oncol Tumor Pharmacother* 1990;7:287–90.
42. Bitton R, Figg W, Venzon D. Pharmacologic variables associated with the development of neurologic toxicity in patients treated with suramin. *J Clin Oncol* 1995;13:2223–9.
43. La Rocca R, Meer J, Gilliatt R. Suramin-induced polyneuropathy. *Neurology* 1990;40:954–8.
44. Braddock P, Hu D, Fan T, Stratford I, Harris A, Bicknell R. A structure-activity of antagonism of the growth factor and angiogenic activity of basic fibroblast growth factor by suramin and related polyanions. *Br J Cancer* 1994;69:890–4.
45. Graham LD, Mitchell SM, Underwood PA. Inhibition of platelet heparitinase by phosphorothioate DNA oligonucleotides. *Biochem Mol Biol Int* 1995;37:239–46.
46. Benezra M, Vlodavsky I, Yayon A, Bar-Shavit R, Regan J, Chang M, Ben-Sasson S. Reversal of basic fibroblast growth factor-mediated autocrine-cell transformation by aromatic anionic compounds. *Cancer Res* 1992;52:5656–62.